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Original article

Expression of the multidrug resistance-associated protein (MRP) gene in primary non-small-cell lung cancer

K. Nooter,[1] F. T. Bosman,[2] H. Burger,[1] K. E. van Wingerden,[1] M.J. Flens,[3] R.J. Scheper,[3] R. G. Oostrum,[1] A. W. M. Boersma,[1] A. van der Gaast[1] & G. Stoter[1]

[1]*Department of Medical Oncology, University Hospital Rotterdam and Rotterdam Cancer Institute (Daniel den Hoed Kliniek);* [2]*Department of Pathology, Erasmus University, Rotterdam;* [3]*Department of Pathology, Free University Hospital, Amsterdam, The Netherlands*

Summary

Background: One of the major problems in the cure of advanced non-small-cell lung cancer (NSCLC) is its lack of response to cytotoxic drug treatment, and the mechanisms underlying this intrinsic drug resistance are unclear.

Patients and methods: We determined the expression of a newly recognised drug resistance gene, the Multidrug Resistance-associated Protein (MRP) gene, in normal lung tissue and in tumour biopsies from 35 surgically resected NSCLCs (11 adenocarcinomas, 24 squamous cell carcinomas). MRP mRNA levels were quantitated by RNase protection assay and expression of the MRP Mr 190,000 glycoprotein was estimated by immunohistochemistry.

Results: Using the MRP-specific monoclonal antibody MRPr1, MRP expression was detected by immunohistochemistry in epithelial cells lining the bronchi in normal lung. In NSCLC approximately 35% of the samples showed elevated MRP mRNA levels. Based on MRP-specific immunohistochemical staining the tumours were divided into 4 groups: 12% were scored as negative (−), 14% showed weak cytoplasmic staining of the tumour cells (±), 40% had a clear cytoplasmic staining (+), and in 34% a strong cytoplasmic as well as membranous staining was observed (++). MRP expression, as estimated by immunohistochemistry, correlated with the MRP mRNA levels quantitated by RNase protection assay (correlation coefficient = 0.745, $p = 0.0009$), with MRP mRNA levels (mean ± SD) of 3.0 ± 1.0 U, 3.5 ± 0.7 U, 7.5 ± 5.9 U, and 19.3 ± 10.7 U, in the (−), (±), (+), and (++) immunohistochemistry expression groups, respectively. Among the squamous cell carcinomas a correlation was observed between MRP staining and tumour cell differentiation: the strongest MRP staining was predominantly found in the well differentiated tumours.

Conclusions: Hyperexpression of MRP is frequently observed in primary NSCLC, especially in the well differentiated squamous cell carcinomas. Further studies are needed to assess the role of MRP in the mechanism of clinical drug resistance in NSCLC.

Key words: MRP, NSCLC, RNase protection assay, immunohistochemistry

Introduction

The prognosis of patients with non-small-cell lung cancer (NSCLC) is poor, with an overall 5-year survival of approximately 15%. One of the major problems in the cure of advanced NSCLC is the insensitivity to cytotoxic drug treatment of both primary tumours and the metastases. Although in vitro studies have revealed different mechanisms of cytotoxic drug resistance in cancer cells (reviewed in [1, 2]), there is no convincing evidence for the involvement of one of these drug-resistance mechanisms in the unresponsiveness of NSCLC to chemotherapy (reviewed in [3]). A new drug resistance gene, the Multidrug Resistance-associated Protein (MRP) gene [4] was recently identified. MRP encodes a 190 kDa membrane bound glycoprotein of 1531 amino acids and is a member of the ATP-binding cassette superfamily of transport proteins [4–6]. Transfection experiments with different eukaryotic expression vectors containing full-length complementary DNAs of the MRP gene have shown that MRP confers multidrug resistance (MDR) to a broad range of natural product drugs, among which are anthracyclines, vinca alkaloids and epipodophyllotoxins [7–9]. As yet, the mode of action by which MRP makes cells MDR is not known. However, the available data suggest that MRP acts both as a plasma membrane efflux drug pump and as a pump for drug accumulation in intracytoplasmic vesicles [9, 10]. By both mechanisms cytoplasmic concentrations of free drug may be reduced to sublethal levels, and in that way MRP would promote cell survival. The association of MRP with clinical drug resistance has not yet been elaborated, and studies on MRP expression in human cancers have just begun [11–15].

In the present study we determined the expression of MRP in normal lung tissue as well as in tumour biopsies from primary NSCLC, using a quantitative RNase protection assay (RPA). MRP protein was determined at tissue level by immunohistochemistry (IHC).

Patients and methods

Tumour samples

Tissue samples were subjected to this research with informed consent of the patients. Samples from normal lung tissue and NSCLC were obtained during surgery, snap-frozen in liquid nitrogen, and stored at −70 °C. In total, tumour biopsies from 35 NSCLC patients (11 adenocarcinomas and 24 squamous cell carcinomas) were analysed.

RNase protection assay

Total RNA was isolated from tissue biopsies by the Lithium-chloride-urea method [16], and quantitated spectrophotometrically at A260. Expression of MRP mRNA was quantitated by RPA as described previously [11, 17]. Briefly, 10 μg of total RNA were hybridised under standard conditions with α-^{32}P labeled RNA transcripts complementary to sequences (nucleotides 239–503) at the 5' end of the MRP mRNA [4, 17]. This probe does not cross-react with the human MDR1 or MDR3 mRNAs [11]. Radiolabeled protected probes were visualised by electrophoresis through a denaturing 6% acrylamide gel, followed by autoradiography. In all assays a human γ-actin probe was included as control for RNA integrity and recovery. All individual experiments included tRNA, as well as RNA isolated from the drug-resistant cell line GLC4/ADR and its parental cell line GLC4 (kindly provided by Dr. E. G.E. de Vries, University of Groningen, Groningen, The Netherlands), as positive and negative controls [17]. Expression levels were quantitated by densitometric scanning of the autoradiographs.

Immunohistochemistry

Cryostat sections of biopsies from NSCLC and normal lung were fixed in cold acetone (10 min, 0 °C), air-dried, and incubated for 60 min at 4 °C with the MRP-specific monoclonal antibody (MAb) MRPr1 as described previously [18]. Antibody binding was detected using alkaline phosphatase-conjugated immunoglobulin (Dako, Copenhagen, Denmark) and alkaline phosphatase substrate using new fuchsine (Dako). The slides were counterstained with haematoxylin and mounted. The specificity of MRPr1 has been documented in detail elsewhere [18]. The MAb is suitable for protein blot analysis, flow cytometry, and immunohistochemical studies, and does not cross-react with the human MDR1 and MDR3 Pgps. Prior to use, MRPr1 was diluted (1:1500) in Tris buffered saline (50 mM Tris pH 7.4) containing normal rabbit serum (10%, w/v), normal goat serum (1%, w/v), and normal human AB serum (1%, w/v). Each assay included the use of an isotype-matched irrelevant MAb (rat IgG2a). Cytospin preparations of the MRP-overexpressing drug-resistant human lung cancer cell line GLC4/ADR and its drug-sensitive parental line GLC4 were used as positive and negative controls, respectively. Staining of the tumour cells was scored on the following semiquantitative scale: negative with only weak staining of the stromal tissues (−); weak cytoplasmic staining of the tumour cells (±); clear cytoplasmic staining of the tumour cells (+); strong cytoplasmic and membranous staining of the tumour cells (++).

Results

MRP mRNA expression

MRP mRNA expression was determined with a sensitive and quantitative RPA in RNA samples isolated from biopsies obtained from normal lung (n = 12) and NSCLC (n = 22). The MRP-overexpressing drug-resistant lung cancer cell line GLC4/ADR and its drug-sensitive parental cell line GLC4 were used in all experiments as positive and negative controls [17], and to compare MRP expression levels in different experiments. Expression levels were quantitated by densitometric scanning of the autoradiographs and the signal obtained with 10 μg of total RNA, isolated from GLC4/ADR cells, was assigned an arbitrary expression level of 100 U (Fig. 1). The lowest, accurate detection limit with this assay appeared to be 1 U. The drug-sensitive parental GLC4 cell line showed an MRP mRNA level of approximately 4 U (Fig. 1) In normal lung biopsies the expression was predominantly low,

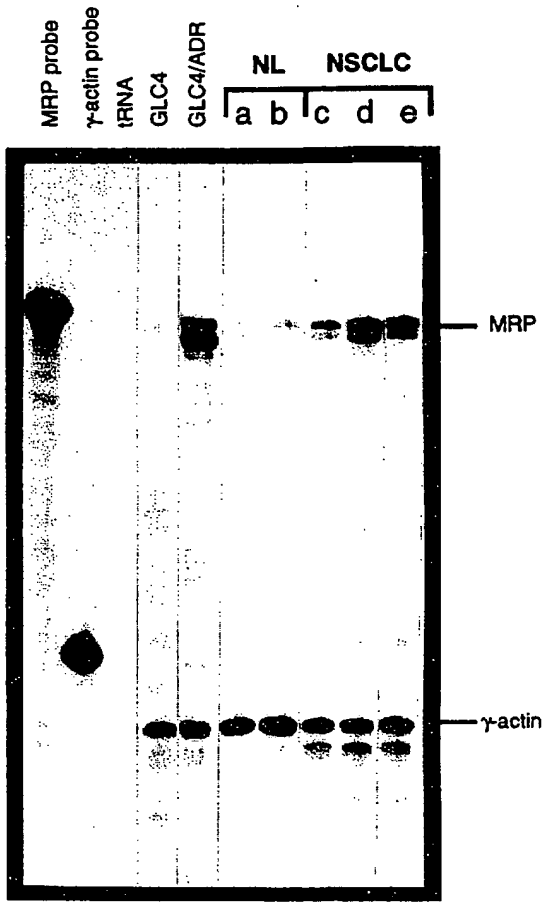


Fig. 1. Analysis of MRP expression by RNase protection assay. Lanes 1 to 3 were loaded with MRP probe, γ-actin probe, and tRNA, respectively. Ten μg of total cytoplasmic RNA from the MRP-hyperexpressing drug-resistant cell line GLC4/ADR and the parental GLC4 line, and biopsies from normal lung tissue and NSCLC were hybridized with MRP- and γ-actin-specific probes. Protected fragments were visualized by gel electrophoresis, followed by autoradiography, and the positions of the specific probes (MRP and γ-actin) are indicated. Photograph is a composite of 3 experiments. The double and triple bands obtained with the MRP probe are probably due to incomplete trimming of the protected probe by RNase A and appeared to be less prominent when a combination of RNase A and RNase T1 was used (not shown). Lane a: normal lung (2 U); lane b: normal lung (5 U); lance C: NSCLC (13 U); lane d: NSCLC (36 U); lane e: NSCLC (30 U).

ranging from 1 to 12 U (mean ± SD, 4.5 ± 3.0 U), an observation in line with those of others, who also found that MRP mRNA is ubiquitously expressed at low levels in normal lung tissues [4, 17]. In all of our NSCLC samples we could detect MRP mRNA. The expression levels among the various tumours ranged from 2 to 36 U (mean ± SD, 10.8 ± 9.8 U) (Fig. 2). Representative examples of RPA on RNA isolated from normal lung and NSCLC samples are shown in Fig. 1.

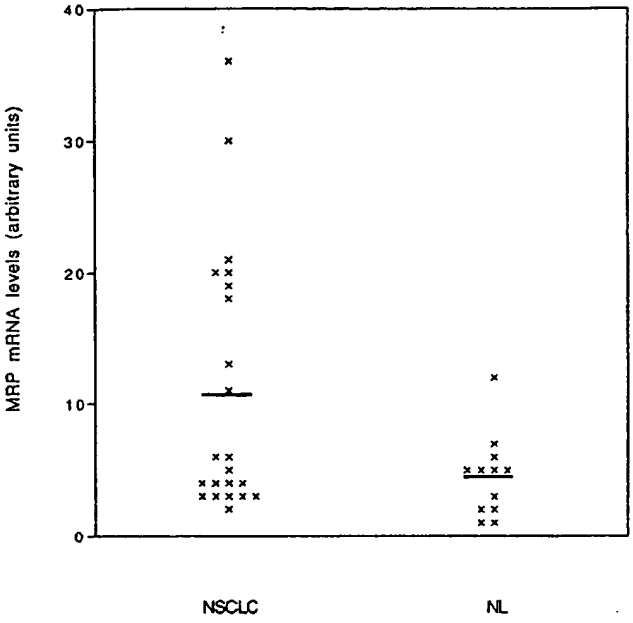


Fig. 2. MRP mRNA expression (in arbitrary units) in normal lung (NL) tissue (n = 12) (mean ± SD, 4.5 ± 3.0 U) and biopsies from NSCLC (n = 22) (mean ± SD, 10.8 ± 9.8 U). MRP mRNA expression levels were determined by RNase protection assay, and expressed in arbitrary units relative to the expression of the MRP-expressing GLC4/ADR cell line, which was set at 100 U.

MRP localisation by immunohistochemistry

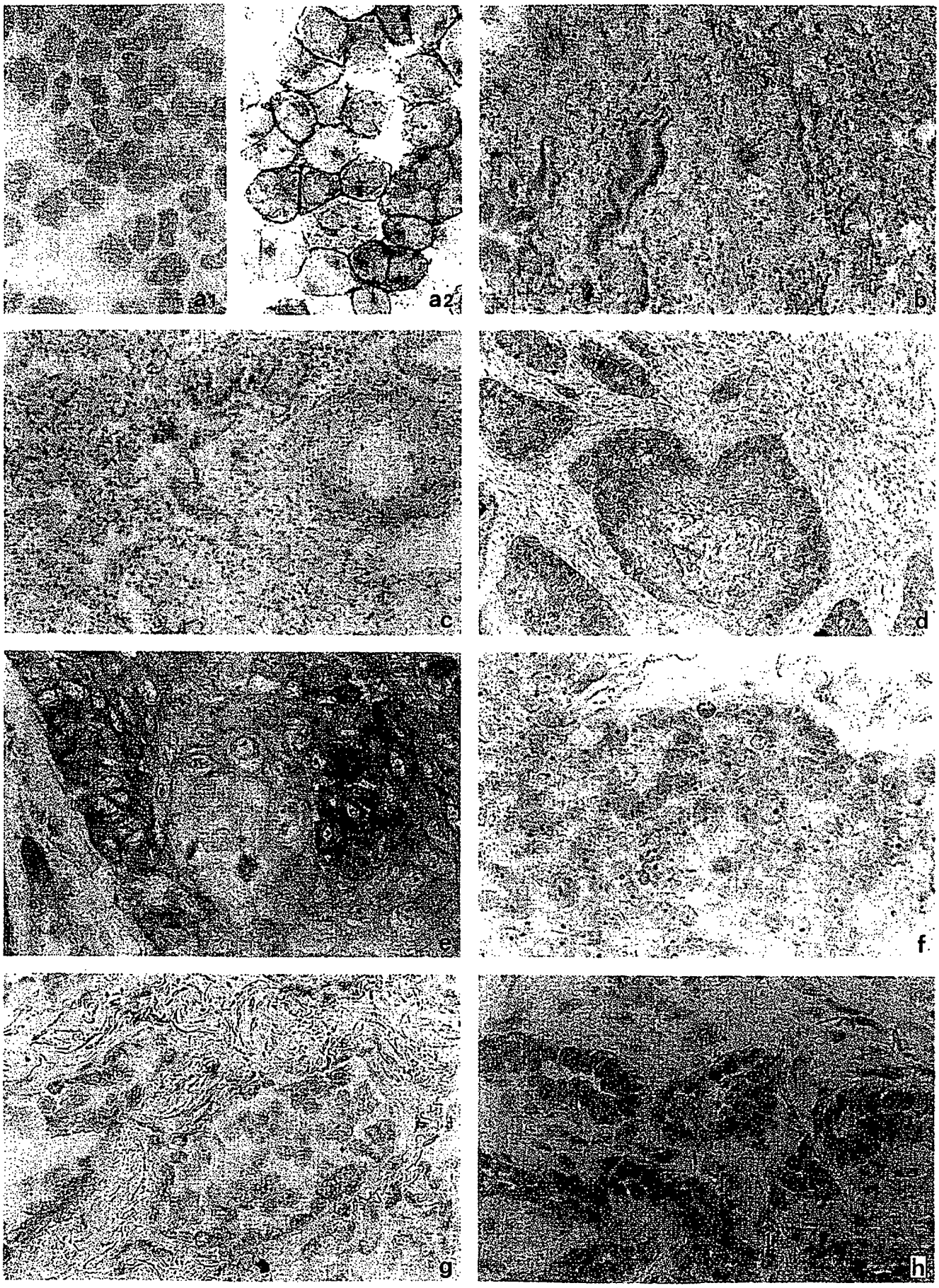
MRP expression at the protein level was determined by IHC with MRPr1 on cryostat sections of normal lung and NSCLC samples. The antibody reacted abundantly with the MRP-positive control cell line GLC4/ADR, whereas in the parental cell line GLC4, no staining or only very weak staining was observed. Figure 3a2 shows MRP staining in GLC4/ADR cells with MRPr1. Membrane staining as well as cytoplasmic staining were observed. The cytoplasmic staining was primarily present as a densely stained spot next to the nucleus, which may correspond to the Golgi network [18]. Epithelial cells lining the bronchi of normal lung clearly stained with MRPr1 (Fig. 3b). The staining specificity of normal bronchial epithelial cells was shown by using an isotype-matched, irrelevant rat MAb (IgG2a subclass) as negative control (Fig. 3c). A total of 35 NSCLC specimens (11 adenocarcinomas and 24 squamous cell carcinomas) were analysed for MRP expression by IHC. Twenty-six of 35 (approximately 75%) of the tumour samples clearly stained with MRPr1, while incubation with the control, irrelevant rat MAb was always negative. The intensity and cellular localisation of the staining varied among the different NSCLC samples, and based on these parameters the tumour samples were qualitatively divided into 4 groups (−, ±, +, ++) with regard to MRPr1 staining (Fig. 4). Four of 35 (approximately 12%) NSCLC specimens were scored as negative (−), 5/35 (approximately 14%) showed weak cytoplasmic staining (±), 14/35 (40%) had clear cytoplasmic staining (+), and in 12/35 (approximately 34%) a strong cytoplasmic as well as membranous staining (++) of tumour cells was observed. The MRP levels of the NSCLCs, estimated by IHC, strongly correlated with the MRP mRNA levels quantitated by RPA (correlation coefficient = 0.745; p = 0.0009) (Fig. 4). The IHC group characterised by undetectable to weak (− and ±) MRP staining had predominantly low MRP mRNA levels, with mean ± SD values of 3.0 ± 1.0 U and 3.5 ± 0.7 U, respectively. The (+) and (++) IHC groups had higher MRP mRNA levels of 7.5 ± 5.9 U and 19.3 ± 10.7 U (mean ± SD), respectively. Representative NSCLC samples incubated with MRPr1 are depicted in Fig. 3d to 3h. A well differentiated squamous cell carcinoma is shown in Fig. 3d (low magnification) and Fig. 3e (higher magnification). The MRPr1 staining is in the cytoplasm as well as at the membrane, and appears to be restricted to the outer cell layers of the tumour. An MRP-negative, poorly differentiated squamous cell carcinoma incubated with MRPr1 is shown in Fig. 3f. A well differentiated adenocarcinoma stained with MRPr1 and an MRP-negative moderately differentiated adenocarcinoma are shown in Fig. 3g and Fig. 3h, respectively.

MRP expression and tumour cell differentiation

We frequently observed high MRP staining with the MRPr1 MAb in the well differentiated squamous cell carcinomas of the lung. Thus, we compared MRP expression, as determined by IHC, with tumour cell differentiation (Table 1). The strongest MRP immunostaining was most frequently found in the well differentiated tumours, whereas the moderately and poorly differentiated tumours stained less intensely for MRPr1.

Discussion

Using in vitro cell lines made resistant to the various classes of cytotoxic drugs, different mechanisms have been elucidated for pleiotropic drug resistance. Among these mechanisms are: enhanced cellular drug efflux by the P-glycoprotein (Pgp) drug pump (reviewed in [1, 2]), decreased or altered topoisomerase IIα [19], and changes in cellular detoxification systems, associated with increased levels of sulphydryl groups and glutathione *S*-transferase activity [20]. Pgp-mediated MDR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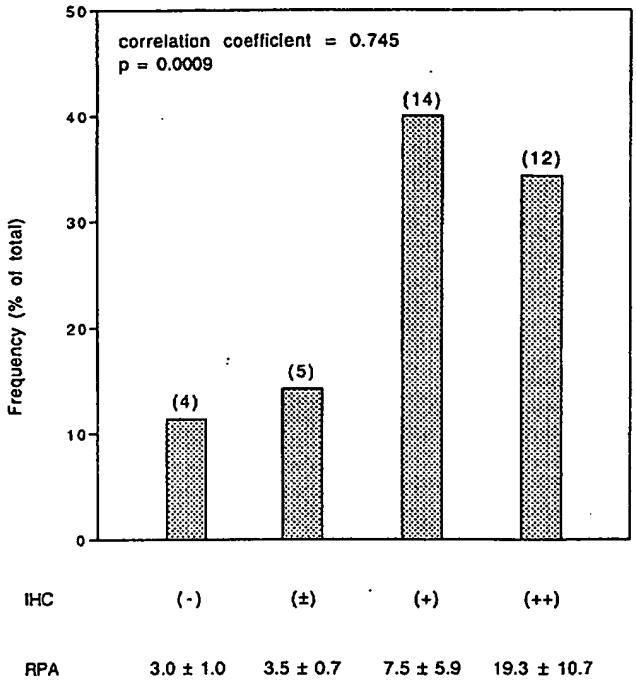

Fig. 4. Distribution of immunohistochemical (IHC) staining of MRP with the MRP-specific MAb MRPr1 in NSCLC. Staining of the tumours was scored according to the following categories: negative (−); weak cytoplasmic staining of the tumour cells (±); clear cytoplasmic staining of the tumour cells (+); strong cytoplasmic and membranous staining of the tumour cells (++). For each category the MRP mRNA levels, as determined by RNase protection assay (RPA) was calculated (mean ± SD). The numbers between brackets represent patient numbers per IHC category. MRP expression, as estimated by IHC, correlated with the MRP mRNA levels, quantitated by RPA (correlation coefficient = 0.745; p = 0.0009).

has thus far been the most intensively studied, both in vitro as well as in patients. Clinical data indicate that Pgp may indeed be involved in the development of drug resistance in certain types of cancer [21–23]. However, for the vast majority of human cancers, including lung cancer, data on the involvement of Pgp in clinical drug resistance are controversial [3, 24, 25]. Apparently in these cancers there are other, yet unknown drug resistance mechanisms.

As a prelude to investigating the possible role of

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Fig. 3. Immunohistochemical staining of MRP expression with the APAAP method, using the MRP-specific MAb MRPr1. The human MRP-positive cell line GLC4/ADR, and the parental GLC4 line served as positive and negative controls, respectively. Cytocentrifuge preparation of parental GLC4 cells (a1) and GLC4/ADR cells (a2) stained with MRPr1 (400×). Cryostat sections of normal lung (b–c), and NSCLC (d–h) stained with MRPr1 or control, irrelevant isotype-matched MAb. Normal lung incubated with MRPr1 (b), or with control, isotype-matched irrelevant MAb (c) (100×), a well differentiated squamous cell carcinoma of the lung stained with MRPr1 (d) (100×) and (e) (400×), an MRP-negative poorly differentiated squamous cell carcinoma of the lung (f) (400×), a well differentiated adenocarcinoma of the lung stained with MRPr1 (g) (400×), and an MRP-negative, moderately differentiated adenocarcinoma of the lung incubated with MRPr1 (h) (400×).

Table 1. Differentiation of squamous cell carcinoma of the lung (n = 24) versus MRP staining with the MRP-specific MAb MRPr1.

Tumour cell differentiation	Immunohistochemical score[a]			
	−	±	+	++
Poorly (n = 4)	1	2	1	
Moderately (n = 10)			8	2
Well (n = 10)			2	8

[a] Staining of the tumours was scored according to the following categories: negative with only weak staining of the stromal tissues (−); weak cytoplasmic straining of the tumour cells (±); clear cytoplasmic staining of the tumour cells (+); strong cytoplasmic and membranous staining of the tumour cells (++).

MRP in clinical drug resistance in lung cancer, we measured MRP expression in 35 surgically resected, untreated primary NSCLC by RPA (n = 22) as well as by IHC (n = 35). With both techniques a substantial number of the NSCLC samples were found to be positive for MRP. With RPA, 7 of 22 (approximately 32%) NSCLC samples had MRP mRNA levels above background (arbitrarily set at 15 U), while using IHC approximately 75% showed clear MRP staining [scored as (+), or (++) on the semiquantitative IHC scale]. The discrepancy between the results obtained by RPA and IHC on MRP expression can readily be explained by the admixture of tumour tissues to normal cells, which, depending on their MRP expression, may influence the MRP levels. It is a general observation that tumour tissues can be infiltrated heavily by normal lymphocytes. However, these fields of infiltrating lymphocytes did not stain with MRPr1 (unpublished results), and thus can only result in underestimation of the MRP mRNA levels. Now that high-affinity MRP-specific MAbs are available for detection of MRP expression at the cellular level [18], heterogeneity of MRP expression within the tumour cell population can be studied with standard IHC techniques. Of note, using IHC we in fact found heterogeneity in MRP expression in NSCLC. Especially in the well differentiated squamous cell tumours, expression of MRP appeared to be restricted to the peripheral layers of the tumours. The keratinizing centres of the well differentiated squamous cell carcinoma cell groups were either negative for MRP or stained very weakly for MRP. In a preliminary study by Twentyman and co-workers [15], in which in situ hybridisation was used for the cellular localisation of MRP mRNA in lung cancer, MRP mRNA was predominantly present in the leading edge of the tumours. This occurrence of MRP expression in cancer cells at the edge of tumours has also been reported for Pgp in colon carcinoma [26]. In that study, localised expression of Pgp at the invasion front of the tumour was associated with local tumour aggressiveness.

Normal epithelial cells lining the bronchi also contained immunoreactive MRP (this study), and MRP mRNA as detected by in situ hybridisation [15]. Apparently, MRP-positive NSCLC develops by prolifera-

tion of MRP-expressing transformed cells. It is of interest that in bronchial epithelium, the reserve (basal) cells, which may differentiate into squamous cells, are especially MRP immunoreactive. However, IHC showed heterogeneity in MRP expression among the NSCLCs, ranging from a complete absence of MRP staining to strong cytoplasmic and membrane staining. The latter was predominantly found in the well differentiated squamous cell lung cancers. Our analysis of the MRP expression in the squamous cell carcinomas suggests a positive correlation between morphological differentiation and MRP expression, in that strong MRP staining was found in the well differentiated tumours and less MRP staining in moderately and poorly differentiated carcinomas. In contrast, another MRP study showed that induction of differentiation in neuroblastoma cell lines results in down-regulation of MRP expression [14]. Whether a similar relationship between MRP expression and differentiation will be apparent in neuroblastomas in vivo remains to be determined.

It was recently shown that MRP is an ATP-dependent outward pump for the endogenous glutathione conjugate leukotriene C_4 and structurally related anionic amphiphilic glutathione conjugates [27–29]. These findings suggest a role for MRP in the cellular elimination of conjugated endogenous substances and xenobiotics from lung epithelium. Related to this putative normal function of MRP is the question of whether MRP accounts for clinical drug resistance in NSCLC. We do not yet have an answer to this question, but the finding that some NSCLC have no detectable MRP expression and that others are highly positive for MRP, calls for a correlative study of MRP expression with clinical outcome of chemotherapy.

The overall conclusion from our study is that expression of MRP, both at the mRNA and the protein levels is observed in NSCLC, especially in the well differentiated squamous cell carcinomas. Further studies are needed to assess the role of MRP in the mechanism of clinical drug resistance in primary NSCLC.

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Correspondence to:
Kees Nooter, Ph.D.
Department of Medical Oncology, Room D328
University Hospital Rotterdam
Dr. Molewaterplein 40
3015 GD Rotterdam
The Netherlands